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14. ABSTRACT

The most significant achievement of this period is the development of a synthetic scheme to produce substantial quantity of our target compound i.e. 1,25-dihydroxyvitamin D3-3-bromoacetate. This is extremely important for the current project and for the future development of this compound for prostate cancer. We have also screened this compound against prostate and kidney cancer cells for its antiproloferative activity. In addition we have developed a mouse xenograft model to test the efficacy of this compound in reducing androgen-sensitive and androgen-insensitive prostate tumors in future studies.

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prostate cancer, vitamin D derivatives, animal models

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Introduction

Therapeutic potential of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in prostate cancer is well-recognized. However, its clinical use has been restricted by its inherent calcemic toxicity. In recent studies we demonstrated that 1α,25-dihydroxyvitamin D_3-3-bromoacetate [$1,25(OH)_2D_3$-3-BE], a derivative of $1,25(OH)_2D_3$ that covalently links $1,25(OH)_2D_3$ inside the ligand-binding pocket of nuclear vitamin D receptor (VDR) is a strong antiproliferative and pro-apoptotic agent in several androgen-sensitive and androgen-refractory human prostate cancer cells. Furthermore, $1,25(OH)_2D_3$-3-BE demonstrated strong anti-prostate tumor effect in athymic mice without toxicity. The goal of this project is to evaluate the translational potential of $1,25(OH)_2D_3$-3-BE as a therapeutic agent for prostate cancer. This will be achieved by determining the efficacy of $1,25(OH)_2D_3$-3-BE in mouse models of human androgen-sensitive and androgen-insensitive prostate cancer, as well as evaluating its molecular mechanisms of action in several *in vitro* studies.

Studies completed/goals achieved during the one year period (Year 1) of the project

Synthesis of $1,25(OH)_2D_3$-3-BE

Success of all the studies included in this project are critically dependent on the availability of $1,25(OH)_2D_3$-3-BE in substantial (milligram) quantities. In the past we synthesized this compound in a multi-step scheme using 1,25-dihydroxy-7-dehydrocholesterol as the starting material as shown in **Figure 1** (R. Ray, S.A. Holick, and M.F. Holick. Synthesis of a photoaffinity-labelled analog of 1,25-dihydroxyvitamin D_3. *Journal of the Chemical Society***,**

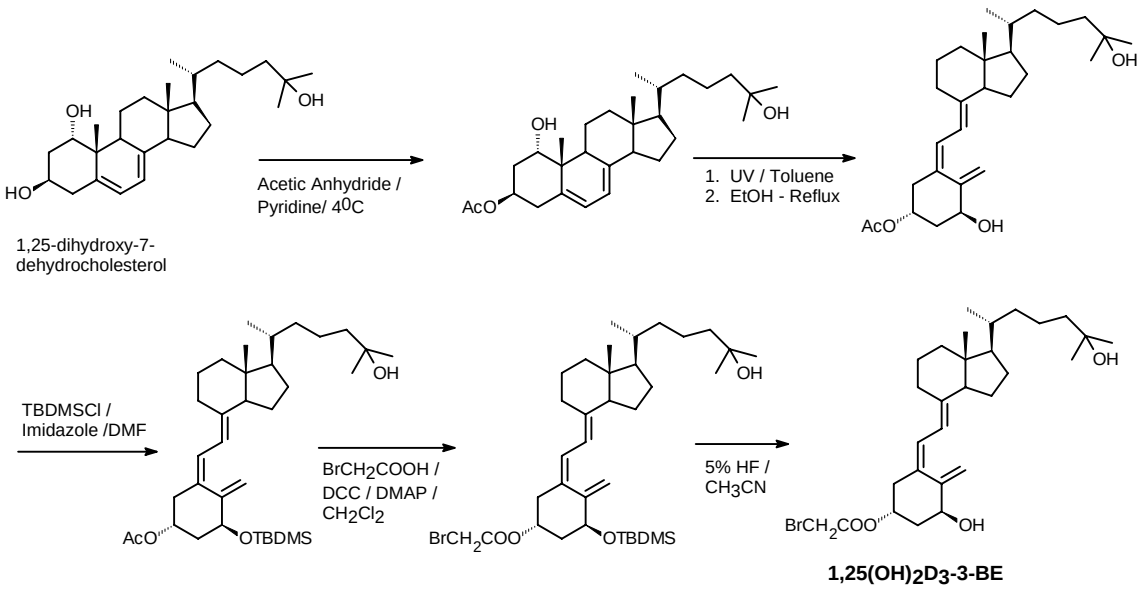

Figure 1: Scheme for the synthsis of $1,25(OH)_2D_3$-3-BE starting with 1,25-dihydroxy-7-dehydrocholestero (Ray et al., Chem. Comm., 1985)l

Chemical Communications 11: 702-703 (1985). However, this starting material is no longer available. Therefore, we had to devise a synthetic scheme for obtaining substantial quantity of $1,25(OH)_2D_3$-3-BE required for our studies by a scheme shown in **Figure 2**.

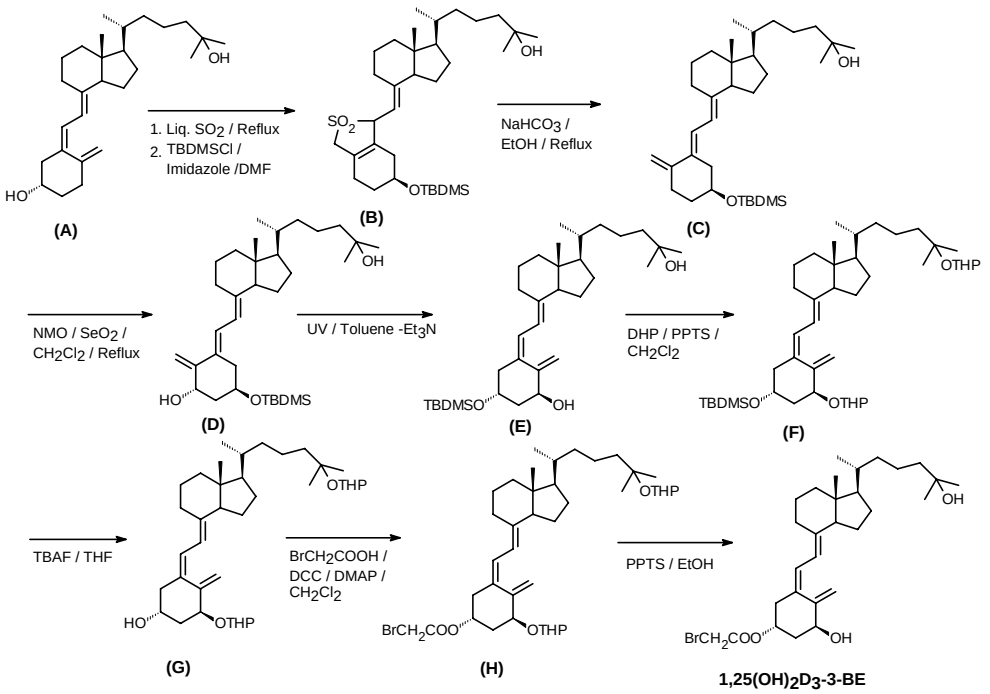

Figure 2: The new scheme for the synthesis of $1,25(OH)_2D_3$-3-BE

Synthetic procedures:

a. 3-TBDMS ether of 25-hydroxyvitamin D_3-SO_2 adduct (B): Approximately 10 ml of SO_2 was condensed (-78^{0}C) in a flask containing 200 mg of 25-hydroxyvitamin D_3 in a flask fitted with a trap that was cooled with dry ice-acetone (-78^{0}C). The yellow solution was refluxed with stirring for 4 hours followed by removal of SO_2 with a stream of nitrogen to produce a foamy solid. The foam was dissolved in 5 ml of anhydrous DMF (distilled fresh from CaO) and TBDMSCl (1.5 X), imidazole (2 X) were added and the solution was stirred at 25^{0}C for 20 hours followed by removal of DMF under vacuo, re-dissolving the residue in EtOAc. The organic solution was washed with water, organic layer was dried over anhydrous $MgSO_4$, and the solution was concentrated under vacuo. The residue was moved on to the next step without further purification.

b. Trans-25-hydroxyvitamin D_3-3-TBDMS ether (C): The crude from the previous step was dissolved in 95% EtOH (10 ml) and $NaHCO_3$ (244 mg) was added. The mixture was refluxed under argon for 90 min followed by addition of brine and extraction of the aqueous solution with EtOAc. The crude reaction product was purified by preparative TLC (silica plates, 1000µ, Analtech) to produce 56.7% of the desired product (C).

c. Trans-1α,25-dihydroxyvitamin D_3-3-TBDMS ether (D): A mixture of (C) (760 mg) and SeO_2 (192 mg) in 15 ml of anhydrous CH_2Cl_2 was refluxed under argon for 30 min followed by cooling to room temperature and addition of a solution of N-methylmorpholine-N-oxide (850 mg) in 15 ml of anhydrous CH_2Cl_2. The mixture was refluxed for an additional 60 min when TLC indicated almost complete reaction, and refluxing was stopped. The mixture was filtered and concentrated under vacuo. The crude reaction product was purified by preparative TLC to produce almost a quantitative amount of the desired product (D).

d. 1α,25-Dihydroxyvitamin D_3-3-TBDMS ether (E): 80 mg of (D), anthracene (10 mg), Et_3N (40 µl) in 10 ml of toluene (in a quartz test tube) was irradiated from a Hanovia medium pressure mercury arc lamp for 75 min. The irradiated solution was concentrated and the crude mixture was purified by preparative TLC (1000 µ plate, 4:1 EtOAc-hexanes, multiple elutions, the desired product (most polar of all the photo-products) was isolated as a gummy liquid in 67% yield.

e. 1α,25-Di-tetrahydropyranyl, 3-TBDMS ether of 1α,25-dihydroxyvitamin D_3 (F): A solution of (E) (35 mg), DHP (60 µl) and a few crystal of PPTS in 1.0 ml of anhydrous CH_2Cl_2 was stirred for two days followed by preparative TLC procedure to produce 34 mg (74%) of the desired product (F).

f. 1α,25-Di-tetrahydropyranyl,1α,25-dihydroxyvitamin D_3 (G): A solution of (F) (17 mg) and 20 µl of TBAF (1M in THF) was dissolved in anhydrous THF (1 ml) and stirred for 20 hours. The reaction mixture was diluted with EtOAc, washed with brine, dried over anhydrous $MgSO_4$, concentrated and purified by preparative TLC to produce quantitative amount of (G).

g. 1α,25-Di-tetrahydropyranyl,1α,25-dihydroxyvitamin D_3-3-bromoacetate (H): A solution of (G) (8 mg), DCC (2.5 X, 8.12 mg), DMAP (catalytic), bromoacetic acid (1.5 X, 3.3 mg) in one ml of anhydrous CH_2Cl_2 was stirred for 20 hours followed by filtration of the mixture, concentration of the filtrate by a stream of nitrogen and preparative TLC purification of the reaction mixture produced quantitative amount of the desired product (H).

h. 1α,25-Dihydroxyvitamin D_3-3-bromoacetate: A solution of (H) (7 mg) was dissolved in 3 ml of AcOH-THF-H_2O (4:2:1) and heated at 50^0C for 20 hours. Volatile matters were removed under a stream of nitrogen and the product was isolated by preparative TLC. Yield of the desired product 1α,25-dihydroxyvitamin D_3-3-bromoacetate was quantitative.

Notes:

1. All the intermediate products were physicochemically characterized by NMR.
2. Purity of the final product (1α,25-dihydroxyvitamin D_3-3-bromoacetate) was determined by HPLC (Altech 10 µ C_{18} column, 5% H_2O-MeOH solvent, 0.5 ml/min, 254 nm detection).
3. NMR spectrum of the final product (1α,25-dihydroxyvitamin D_3-3-bromoacetate) is included in Figure 3.

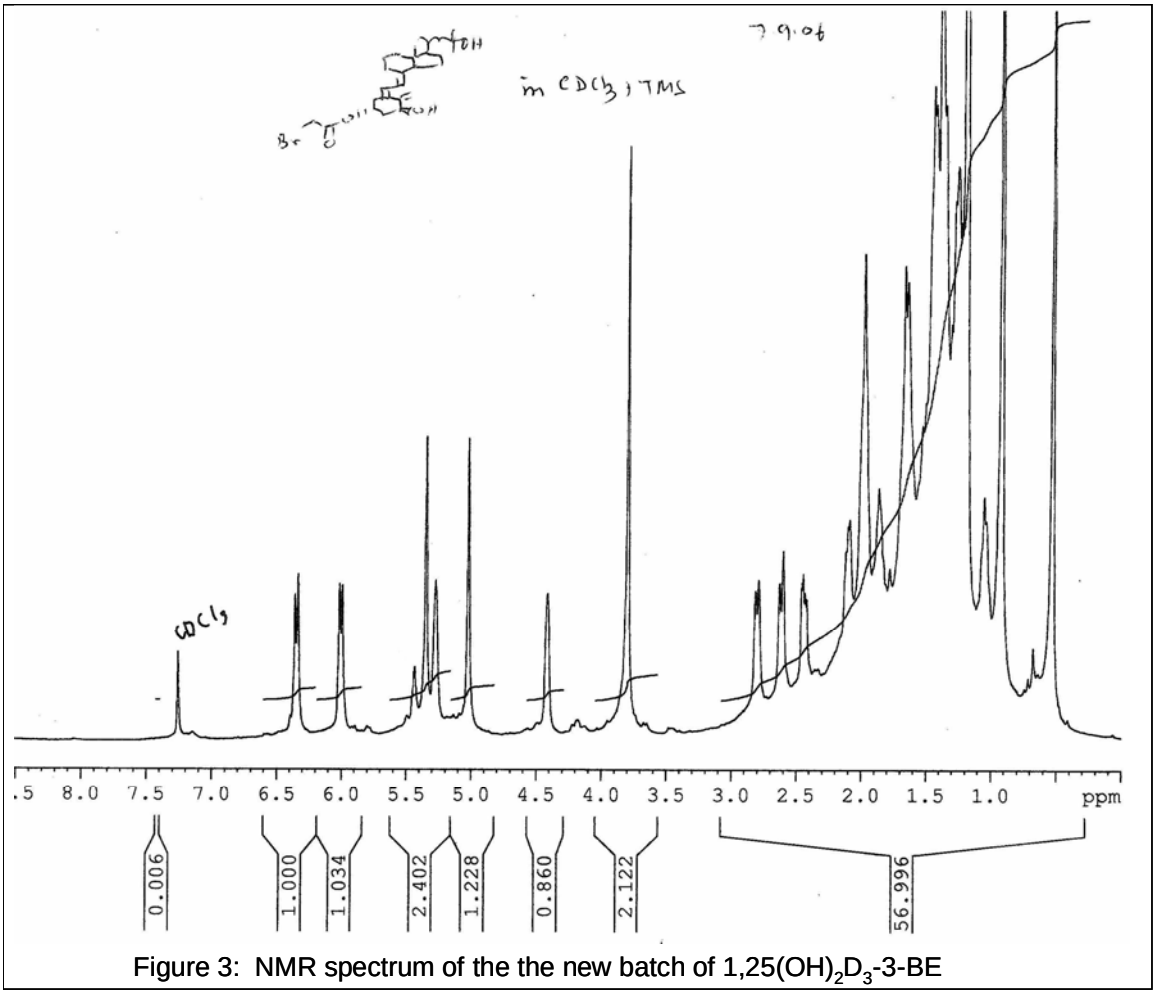

Figure 3: NMR spectrum of the the new batch of $1,25(OH)_2D_3$-3-BE

Comparison of the new batch of $1,25(OH)_2D_3$-3-BE with the old batch

Since we synthesized our compound ($1,25(OH)_2D_3$-3-BE) by a different synthetic scheme (Figure 1 vs. Figure 2) we were compelled to compare the in vitro cellular activity of the same compound obtained from different synthetic schemes.

Determination of the antiproliferative activity of $1,25(OH)_2D_3$-3-BE (new compound and old compound) in Caki-1 kidney cancer cells by ^{3}H-thymidine incorporation assay

Procedure: Caki-1 cells (ATCC, Manasas, VA) were grown in McCoy's 5A media with 5% fetal bovine serum (FBS) according to manufacturer's specifications and then plated onto 24-well plates and grown to approximately 60% in the same media containing 5% FBS followed by overnight serum-starvation and treatment with $10^{-6\text{-}7}$M of $1,25(OH)_2D_3$-3-BE (old or new batch) or $1,25(OH)_2D_3$ or ethanol (control). Compounds were dissolved in minimum amount of ethanol and required aliquots of the ethanolic solutions of the compounds were added to McCoy's 5A media with 5% FBS. Volume of ethanol was adjusted so that its concentration was 0.1% v/v in the media.

^{3}H-thymidine-incorporation assay: After the treatment media was removed from the wells and replaced with media containing ^{3}H-thymidine (0.1μCi) per well, and the cells were incubated for 3 hours at 37^0C. After this period media was removed by aspiration and the cells were washed thoroughly (3 X 0.5 ml) with PBS. Then ice-cold 5% perchloric acid solution (0.5 ml) was added to each well and the cells were incubated on ice for 20 minutes. After this incubation, perchloric acid was removed by aspiration, replaced with 0.5 ml of fresh perchloric

acid solution and the cells were incubated at 70^0C for 20 minutes. Solution from each well was mixed with scintillation fluid and counted in a liquid scintillation counter. There were eight (8) wells per sample, and statistics were carried out by Student's t test. Results are expressed as

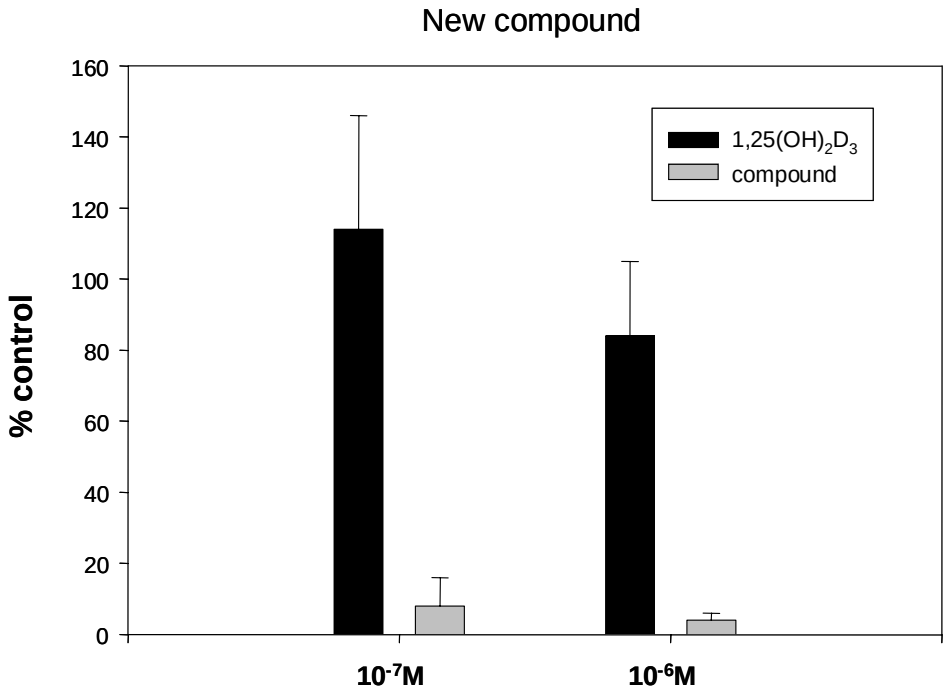

Figure 4: ^{3}H-Thymidine incorporation assay of Caki-1 cells with 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_3$-3-BE (New Batch)

percent incorporation of radioactivity versus ethanol-control.

Results: Results of this assay are shown in Figures 4 and 5. In this cell line 1,25(OH)$_2$D$_3$-3-BE was a much stronger antiproliferative agent than 1,25(OH)$_2$D$_3$ in both dose levels tested ($10^{-6\text{-}7}$M); and both 'new' and 'old' batches of 1,25(OH)$_2$D$_3$-3-BE were same in terms of their

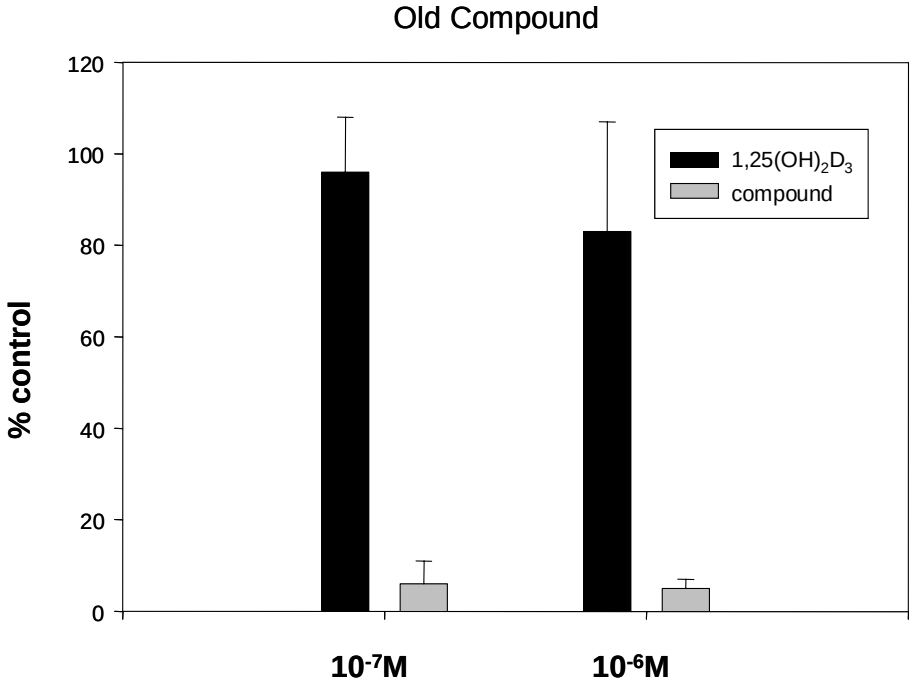

Figure 5: ^{3}H-Thymidine incorporation assay of Caki-1 cells with 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_3$-3-BE (Old Batch)

antiproliferative activity.

Determination of the antiproliferative activity of the 'new batch' of $1,25(OH)_2D_3$-3-BE' in DU-145 human androgen-insensitive prostate cancer cells

DU-145 cells were grown in DMEM media containing 5% FBS and plated on to 24-well plates. The cells were grown to approximately 60% confluence and treated with $10^{-6\text{-}7}$M of $1,25(OH)_2D_3$-3-BE (new batch) or $1,25(OH)_2D_3$, followed by ^{3}H-thymidine incorporation assay as described earlier.

Results: Results of this assay (not shown) demonstrated that the 'new batch' of $1,25(OH)_2D_3$-3-BE' is strongly antiproliferative towards DU-145 prostate cancer cells.

Studies to develop a mouse xenograft model of androgen-insensitive human prostate tumor

Once our compound ($1,25(OH)_2D_3$-3-BE) became available in significant quantity we initiated a study to develop a model of androgen-insensitive human prostate tumor in a mouse xenograft model.

Procedure: Six (6) weeks old male athymic mice, weighing approximately 20 gm (Charles River) were kept on normal animal diet and water *ad libitum*. DU-145 human androgen insensitive prostate cancer cells were grown in DMEM media containing 5% FBS. When the cells grew to confluence they were trypsinized and centrifuged. The cell pellet was suspended in PBS. An aliquot was counted for cell-number. Approximately 10^6 cells/ 100 µl of suspension was injected (with a 26 gauge needle) under the skin in the flank of the mice. Appearance of tumor was detected within four (4) days. Palpable tumor with an approximate size of 1 mm^3 was obtained within two weeks post-injection.

Plans for the immediate future: As described above we have developed a mouse xenograft model of androgen-insensitive prostate tumor. Currently we are in the process of developing a study to determine maximum tolerated dose (MTD) of $1,25(OH)_2D_3$-3-BE in a mouse model. Once that is established we will order a large number of athymic mice for our proposed efficacy study of $1,25(OH)_2D_3$-3-BE.

During the past one year our efforts with $1,25(OH)_2D_3$-3-BE and a related has generated three (3) abstracts and one manuscript that are included in the appendix.

KEY RESEARCH ACCOMPLISHMENTS

- Synthesized substantial quantity of $1,25(OH)_2D_3$-3-BE by a new synthetic route (reportable separately in a synthetic/,edicinal chemistry journal) that will be required for the *in vitro* and *in vivo* studies included in this project.

- Physicochemically determined the structural and chemical homogeneity of the compound ($1,25(OH)_2D_3$-3-BE).

- Compared the antiproliferative activity of the 'new batch' of $1,25(OH)_2D_3$-3-BE with the 'old batch' in an antiproliferative assay in Caki-1 kidney cancer cell-line.

- Determined the antiproloferative activity of the ‘new batch’ of $1,25(OH)_2D_3$-3-BE in DU-145 human androgen-insensitive prostate cancer cells.

- Established a mouse xenograft model androgen-insensitive prostate tumor model in our laboratory.

- Extended our anti-cancer studies of $1,25(OH)_2D_3$-3-BE beyond prostate cancer to kidney cancer. This resulted in a manuscript (included in the appendix) that has been provisionally accepted for publication in *Molecular Cancer Therapeutics.*

REPORTABLE OUTCOME

During the past one year our efforts with $1,25(OH)_2D_3$-3-BE and a related has generated three (3) abstracts and one manuscript that are included in the appendix.

CONCLUSION

Our effort for the past one year has established the groundwork for the current year and beyond to develop $1,25(OH)_2D_3$-3-BE and related compounds for prostate and other cancers.

APPENDIX

Meeting Abstract 1:

Abstract:

Vitamin D and Cancer. Brown University Symposium on Vitamin D. Seekonk, MA, September, 2005

Abstract:

VDR-alkylating derivatives of vitamin D for potential cancer-therapy. Rahul Ray, Ph.D. Boston University School of Medicine, Boston, MA

Protein and DNA-alkylating agents are common in cancer therapy. But, majority of them are non target-specific causing systemic toxicity. We hypothesized that derivatives of 25-OH-D_3 and 1,25$(OH)_2D_3$ that covalently attach to the ligand binding pocket of VDR in the target cells might reduce the catabolic process, lower the therapeutic dose and diminish toxicity. Recently we observed that bromoacetate derivatives of 25-OH-D_3 and 1,25$(OH)_2D_3$, that alkylate the ligand-binding site of VDR, produced significantly stronger antiproliferative effect than the hormone in several cancer cells. The effect was particularly strong in a host of androgen sensitive and androgen insensitive prostate cancer cells, as well as kidney and bladder cancer cells. The reason for this cancer-specific effect is unknown at this point. In vivo studies in nude mice, bearing DU-145 inoculated prostate tumor demonstrated strong anti-tumor activity of the abovementioned compounds without toxicity. In conclusion, VDR-alkylation is a potentially important method for developing vitamin D-based therapeutic agents for cancer.

Meeting Abstract 2:

Endocrine Society National Meeting, Boston, MA, June 2006

Abstract:

A vitamin D receptor-alkylating derivative of 1,25-dihydroxyvitamin D_3 induces apoptosis in prostate and kidney cancer cells and displays anti-tumor effect in a DU-145 mouse xenograft model. Rahul Ray, James M. Lambert, Sibaji Sarkar and Kelly S. Persons. Boston University School of Medicine, Boston, MA, and University of Colorado Health Sciences Center, Aurora, CO.

Currently no therapy is available for prostate cancers that fail to respond to androgen therapy. Most human prostate cancer cells contain the receptor for 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$); and numerous studies have shown that prostate cancer cells respond to 1,25$(OH)_2D_3$ by enhancing differentiation and decreasing proliferation. Furthermore, several epidemiological studies have suggested a strong connection between prostate cancer mortality and exposure to sunlight, which is required for the production of vitamin D in the body. These findings strongly support the use of vitamin D-based agents in prostate cancer. However,

inherent toxicity of $1,25(OH)_2D_3$ in therapeutic doses has limited its use and spurred a strong interest in developing less toxic analogs of $1,25(OH)_2D_3$ for cancer-therapy.
In recent studies we demonstrated that 1α,25-dihydroxyvitamin D_3-3-bromoacetate ($1,25(OH)_2D_3$-3-BE), a derivative of $1,25(OH)_2D_3$ that covalently links $1,25(OH)_2D_3$ inside the ligand-binding pocket of nuclear vitamin D receptor (VDR) strongly inhibits the growth of LNCaP, PC-3, DU-145 and LAPC-4 prostate cancer cells and induces apoptosis as determined by FACS analysis. We also demonstrate that $1,25(OH)_2D_3$-3-BE modulates the message for 1,25-dihydroxyviamin D_3-24-hydroxylase, a key gene in the vitamin D pathway; and the signal is obliterated by ZK 159222, a VDR-antagonist. Furthermore, in a recent preliminary study we observed that $1,25(OH)_2D_3$-3-BE produced strong anti-prostate tumor effect in DU-145 mouse xenograft model without toxicity.
In addition to prostate cancer cells we recently observed that $1,25(OH)_2D_3$-3-BE strongly inhibits the growth and induces apoptosis in two human kidney cancer cell lines.
Protein and DNA alkylating agents are standard therapeutic agents for cancer. But, these drugs are non-specific and, produce significant to severe side effects, particularly at therapeutic doses. On the other hand, affinity alkylating derivatives of drugs and drug-candidates specifically alkylate the substrate/ligand-binding sites of target enzymes/receptors, thereby they can potentially modulate the biological property associated only with the target molecules; thereby lowering the required dose and toxicity. We hereby report that a derivative of $1,25(OH)_2D_3$, that alkylates the hormone-binding pocket of VDR demonstrates a strong translational potential as non-toxic therapeutic agents for prostate and renal cancers.

Meeting Abstract 3:

International Conference on Biotechnology and nanotechnology. Kolkata, India, March, 2006

Abstract:

Nuclear transcriptional factors as molecular targets for drug-discovery and delivery: bench to bedside. Rahul Ray, Ph. D, Boston University School of Medicine, Boston, Massachusetts, USA

In the past discovery of drugs for various diseases used to be a 'hit or miss' process. It is no longer the case. Advancement of our knowledge regarding biology of diseases, and particularly of the intricate biochemical processes have given us appropriate means of carefully choosing the specific target for any particular disease. This has been aptly complemented by a plethora of highly efficient modern techniques, such as x-ray crystallography, nuclear magnetic resonance imaging, computer simulation, combinatorial chemistry, nanotechnology etc.

Research from our group has focused primarily on cancer drug-discovery and its delivery to the desired target. Despite a well-coordinated effort by scientists in the world over; and availability of a sea of knowledge, cancer in general has remained a scourge and a major killer. It is now realized that each malignancy is unique. In addition, our gender, socio-economic status, and life style have a profound effect on cancer development, growth and mortality.

In our effort we focused on two small naturally occurring molecules (vitamin D and estrogen) and their cognate transcriptional factors/nuclear hormone receptors, namely vitamin D receptor (VDR) and estrogen receptor (ER). Vitamin D is strongly implicated in many cancers, while estrogen is implicated in malignancies of breast, ovary and endometrium. On the other hand

VDR and ER are proteins that are abundant in the nucleus of the cancer cells; and act as highly efficient scavengers of vitamin D and estrogen. This binding/scavenging process is ultimately manifested in the biological properties of vitamin D and estrogen. In layman's terms a key fits perfectly in a unique key-hole to open the door to a room with hidden treasures. Allegorically key is the small molecule (vitamin D, estrogen), while the door is the hormone receptor (ER, VDR) and access to the hidden treasure is the property/manifestation of the property of the key

We developed a computer simulated model of the three-dimensional structure of VDR to design novel analogs of vitamin D (the key) that cross-links to the ligand-binding site (the key hole) of VDR (the door). We postulate that such a process might enhance the biological potency of the parent hormone. As support for this hypothesis we observed that these compounds strongly inhibit the growth of several cancer cells; and the effect is strongest in prostate, kidney and bladder cancer cells. Under treatment, these cancer cells undergo programmed cell-death (apoptosis) as evidenced by key signatures of cell-death including caspase-activation and DNA-fragmentation, demonstrating the potential of these vitamin D compounds to treat these cancers.

Specially bred rodents such as mouse and rat are commonly used animal models to test efficacy of an experimental drug. In other words human cancer cells, grown in petri dish, can be injected under the skin of these animals to grow tumors (xenograft model). Then the animals can be treated with an experimental drug to determine its efficacy in reducing/eliminating tumor. In essence these animals act as test tubes in a chemical/biochemical laboratory, except these animals represent a truly living system close to humans.

The following manuscript has been provisionally accepted for publication in Molecular Cancer Therapeutics

A vitamin D receptor-alkylating derivative of 1α,25-dihydroxyvitamin D_3 exerts antiproliferative and pro-apoptotic effects in human kidney cancer cells.

James R. Lambert[2], Sibaji Sarkar[1], Christian D. Young[2], Kelly S. Persons[1], Douglas V. Faller[1], Michael F. Holick[1], and Rahul Ray[1,*]

[1]Department of Medicine, Boston University School of Medicine, Boston, MA; [2]Department of Pathology, University of Colorado Health Science Center, Aurora, CO

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Short Title: An alkylating 1,25-dihydroxyvitamin D_3 derivative for renal cancer

Abstract

Renal cell carcinoma is a fatal disease with very few therapeutic options. 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the vitamin D hormone has shown strong promise as a general antiproliferative agent in several maliganacies, yet its therapeutic application has been limited by its inherent toxicity, particularly at pharmacological dose. We have developed 1α,25-dihydroxyvitamin D_3-3-bromoacetate ($1,25(OH)_2D_3$-3-BE), a derivative of $1,25(OH)_2D_3$ that covalently and specifically links to the hormone-binding pocket of vitamin D receptor (VDR) in target cells. We hypothesize that such a process might decrease/eliminate catabolic degradation, and enhance the efficacy of $1,25(OH)_2D_3$-3-BE as an antiproliferative agent. In this communication we report that $1,25(OH)_2D_3$-3-BE causes strong growth-inhibition in A498 and Caki 1 human kidney cancer cells with a significantly stronger efficacy than an equivalent concentration of $1,25(OH)_2D_3$. In addition, we reveal that $1,25(OH)_2D_3$-3-BE induces apoptosis in A498 cells, involving Akt-signaling pathway. Furthermore, we demonstrate that antiproliferative activity of $1,25(OH)_2D_3$-3-BE is correlated with the activation of 1α,25-dihydroxyvitamin D_3-24-hydroxylase gene, qualitatively similar to $1,25(OH)_2D_3$. This effect is obliterated by co-treatment with ZK 159222, an inhibitor of $1,25(OH)_2D_3$-mediated gene expression. In summary, results described in this communication demonstrate that $1,25(OH)_2D_3$-3-BE, a derivative of $1,25(OH)_2D_3$ induces strong antiproliferative effect in kidney cancer cells via VDR-mediated signaling pathway. Therefore, $1,25(OH)_2D_3$-3-BE has a strong translational potential as a therapeutic agent in renal cell carcinoma.

Introduction

Renal cell carcinoma (RCC) accounts for approximately 90-95% of all kidney cancer, which comprises approximately 3% of all adult malignancies with over 25,000 new cases and an estimated 8,000 deaths in the United States in 2006 (1). RCC has been increasing at a rate of approximately 3% a year in the United States and Europe and the overall death rate has not changed. Furthermore, approximately 50% of localized RCC develops into metastatic disease within a relatively short time frame (2). In addition, RCC is manifested by lack of early signs of neoplasm, and resistance to standard chemo- and radiation therapy (2, 3). Current therapeutic options include radical nephrectomy in early stages and immunotherapy in late and metastatic stages with some success. Anti-angiogenic and Raf-kinase inhibiting small molecules have also shown promise in treating RCC (4-6). Clearly, effective therapy for this deadly disease is urgently needed.

Numerous epidemiological studies have demonstrated the importance of dietary vitamin D in preventing various cancers (7-9). In addition, the therapeutic potential of 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the biologically active metabolite of vitamin D and its analogs in cancer is well-documented (10). However, inherent calcemic toxicity of this hormone, particularly at therapeutic doses, has prevented its general use as an anticancer agent. Thus, development of vitamin D analogs exhibiting potent antiproliferative activity and reduced systemic toxicity has become an active area of research.

For the past several years our laboratory has been engaged in developing novel analogs of $1,25(OH)_2D_3$ and its pre-hormonal form, 25-hydroxyvitamin D_3 (25-OH-D_3) that covalently and specifically label the ligand-binding pocket of the nuclear receptor for $1,25(OH)_2D_3$, the vitamin D receptor (VDR). Recently we reported that 1α,25-dihydroxyvitamin D_3-3-bromoacetate ($1,25(OH)_2D_3$-3-BE) and 25-hydroxyvitamin D_3-3-bromoacetate (25-OH-D_3-3-BE), VDR-alkylating derivatives of $1,25(OH)_2D_3$ and 25-OH-D_3 respectively, are more potent antiproliferative agents than $1,25(OH)_2D_3$ in human cancer cell lines including hormone-sensitive and hormone-insensitive prostate cancer cell lines (11,12). Furthermore, we recently observed that $1,25(OH)_2D_3$-3-BE and 25-OH-D_3-3-BE significantly reduce tumor size in a mouse xenograft model of hormone insensitive prostate tumor (manuscripts in preparation).

In the present study we compared the growth-inhibitory property of $1,25(OH)_2D_3$-3-BE with $1,25(OH)_2D_3$ in two renal cancer cells and examined the molecular mechanism of its cellular properties. Results of these studies and potential therapeutic application of $1,25(OH)_2D_3$-3-BE in renal cell carcinoma are presented.

Materials and Methods

Materials. $1,25(OH)_2D_3$-3-BE was synthesized according to our published procedure (13). A498 (HTB-44) and Caki 1 (HTB-46) cell lines were purchased from ATCC (Manassas, VA). ZK 159222 was obtained from Schering (Berlin, Germany).

Cellular Proliferation Assay. A498 and Caki 1 cells were grown in MEM and McCoy's 5A media respectively with 5% FBS according to manufacturer's specifications and plated into 96-well plates (500-1000 cells/well). The following day cells were treated with various doses of $1,25(OH)_2D_3$-3-BE or $1,25(OH)_2D_3$ or ethanol (vehicle) (in media containing 5% FBS). The cells were dosed a total of three (3) times on alternate days. After seven (7) days, MTT solution (10 µl) was added to each well, and the plates were incubated at 37^0C for 3 hours followed by the addition of detergent solution (100 µl/well). The plates were incubated at 25^0C for 15 hr and then absorbance was read with a microplate reader.

Measurement of Apoptosis. Apoptosis measurements were carried out using the apoptosis kit (BD Biosciences) according to the manufacturer's instructions. Briefly, A498 kidney cancer cells were grown in DMEM media with 10% FBS to 60-70% confluence, and then were incubated (in media with 10% FBS) with 10^{-6}M of either $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$-3-BE for 6 hours in a cell incubator. The control cells contained equivalent amount of ethanol as vehicle control. The cells were harvested, washed with PBS and then once with binding buffer. Cells were re-suspended in 100 ul of 1X binding buffer and then stained with Annexin-FITC and Propidium Iodide (PI) for 15 minutes in the dark, followed by dilution with 1X binding buffer, according to the manufacturer instructions. Fluorescence was measured in a FACS analyzer with FITC in the X-axis and PI in the Y-axis in log scale.

Western anlysis of phopho-Akt. A498 cells were plated at $3x10^5$ cells/well in a six well dish. 16 hr later the cells were treated with $5x10^{-7}$ M of $1,25(OH)_2D_3$-3-BE, $5x10^{-7}$ $1,25(OH)_2D_3$ or ethanol control. 24 hrs later the cells were washed with PBS, scraped in PBS and centrifuged. Total cellular extracts were prepared by resuspending the cell pellets in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5) containing 50 mM NaF, 1 mM Na_3VO_4 and protease inhibitors (Protease Inhibitor Cocktail, Cat # P8340, Sigma-Aldrich, St. Louis, MO). After 10 min on ice, the extracts were cleared by micro-

centrifugation for 10 min, the supernatants transferred to new tubes and protein concentration of each extract determined by Bradford assay. Samples were separated on 4-12% MES NuPAGE gels (Invitrogen, Carlsbad, CA) and transferred to PVDF membrane. The blot was developed with mouse anti-phospho Akt (Ser 473, Cell Signaling, MA). The blot was stripped and re-probed for total Akt as a loading control with rabbit anti-Akt as a loading control (Cell Signaling, MA). Signals were detected by enhanced chemiluminescence (Perkin Elmer, Boston, MA) and autoradiography. In a parallel assay cells were grown and treated in full serum (10% FBS).

RT-PCR. A498 cells were plated at 3 x 10^5 cells per well of a six well dish. The following day, the cells were treated with various concentrations of 1,25$(OH)_2D_3$ or 1,25$(OH)_2D_3$-3-BE. Sixteen (16) hours later, total RNA was prepared by Trizol extraction (Invitrogen, Carlsbad, CA). One microgram of total RNA was subjected to reverse transcription using moloney murine leukemia virus (MMLV) reverse transcriptase under standard conditions. Following cDNA synthesis, 1/10th of the reaction was subjected to PCR using gene specific primers to both 24-hydroxylase and beta-actin. The products were analyzed on a 1% agarose gel. In a separate experiment A498 cells were treated with 10^{-8}M of either1,25$(OH)_2D_3$-3-BE alone or in the presence of various concentrations of ZK 159222, an inhibitor of 1,25$(OH)_2D_3$ (14).

Results and Discussion

Numerous studies have registered strong promise of 1,25$(OH)_2D_3$ as a therapeutic agent for various cancers. However, its clinical use has been limited by the risk of toxicity related to hypercalcemia, hypercalciuria, and significant loss of body weight. Attempts to address the toxicity-issue have taken two approaches. In the first, combination of 1,25$(OH)_2D_3$ with standard chemotherapeutic agents are being investigated to harness synergy between these compounds. For example, Trump, Johnson and their coworkers have carried out several studies to demonstrate that toxic effects of 1,25$(OH)_2D_3$ can be mitigated by combination with dexamethasone or paclitaxel (15-19). The second approach involves the development of less toxic analogs of 1,25$(OH)_2D_3$ that maintain potent antiproliferative activity. These compounds are being developed as potential therapeutic agents. EB-1089, one such analog is currently in clinical trials for hepatocellular and pancreatic carcinomas (20, 21).

There is a serious paucity of information about the effect of 1,25$(OH)_2D_3$ and its analogs in renal cancer. In 1986 Nagakura *et al*. demonstrated that 1,25$(OH)_2D_3$ and some of its metabolites inhibited the growth of the renal cancer cell line KU-2 (22). In addition, Fuzioka et al. demonstrated that 1,25$(OH)_2D_3$ inhibited the growth of murine Renca renal cancer cell line-induced tumor in a mouse model (23). These results suggest potential utility of 1,25$(OH)_2D_3$ and its analogs in treating renal cancer.

Therapeutic potency of 1,25$(OH)_2D_3$ and its analogs depends on two factors, namely their ability to interact with VDR in target cells; and their bioavailability and pharmacokinetic properties. Interaction between 1,25$(OH)_2D_3$ and VDR is an equilibrium process. Therefore, in the steady state a finite amount of free 1,25$(OH)_2D_3$ (not bound to VDR) is always present in the equilibrium mixture, which undergoes rapid catabolic degradation (as shown in the cartoon in **Figure 1, Upper Panel**). From a therapeutic standpoint such catabolic degradation (of 1,25$(OH)_2D_3$) is met with high pharmacological doses that cause toxicity. In contrast, if catabolic degradation of 1,25$(OH)_2D_3$ is reduced/eliminated, its therapeutic potency can be enhanced significantly.

Chemical structures of 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$–3-BE are shown in **Figure 1**. 1,25$(OH)_2D_3$–3-BE is a derivative of 1,25$(OH)_2D_3$ that contains a chemically reactive bromoacetate group which can react with a nucleophilic amino acid. Earlier we reported that 1,25$(OH)_2D_3$–3-BE cross-links to the ligand-binding pocket of VDR by reacting with a single cysteine residue in the binding pocket (24). We argued that, 1,25$(OH)_2D_3$-3-BE, once covalently linked to VDR, can not exit the binding pocket. Therefore, its interaction with VDR becomes an irreversible process. Furthermore, covalently linked 1,25$(OH)_2D_3$–3-BE is prevented from

interacting with catabolic enzymes (as shown in the cartoon in **Figure 1**, **Bottom Panel**). We argued that such a process will increase the effective concentration of $1,25(OH)_2D_3$ in tandem with $1,25(OH)_2D_3$ –3-BE.

Another possible benefit of $1,25(OH)_2D_3$ –3-BE is that it can potentially 'titrate' and engage all VDR molecules due to the kinetic nature of the alkylation process (**Figure 1, Bottom Panel**). It is an important consideration in cases where VDR level is low. For example, in a recent study Trydal *et al.* determined VDR level in 23 primary renal cell carcinomas and compared with autologous normal kidney tissue; and reported that mean value for the renal cell carcinomas is approximately three times less than autologous normal kidney tissue (25). Therefore, covalent labeling of VDR by $1,25(OH)_2D_3$ –3-BE may compensate for lower VDR-copy number in some renal cancer cells, thereby enhancing the efficacy of this $1,25(OH)_2D_3$ derivative.

$1,25(OH)_2D_3$-3-BE is more potent than $1,25(OH)_2D_3$ in inhibiting the growth of renal carcinoma cells. To compare the antiproliferative potential of $1,25(OH)_2D_3$-3-BE versus $1,25(OH)_2D_3$ we treated A498 and Caki 1 kidney cancer cells with various doses of $1,25(OH)_2D_3$-3-BE and $1,25(OH)_2D_3$ and determined cell-viability. In A498 cells, treatment with 10^{-6}M $1,25(OH)_2D_3$-3-BE almost completely inhibited the growth of the cells, while an equivalent amount of $1,25(OH)_2D_3$ inhibited the growth by approximately 10%. At 10^{-7} M and 10^{-8} M dose levels of $1,25(OH)_2D_3$-3-BE the antiproliferative effect is significantly stronger than an equivalent concentration of $1,25(OH)_2D_3$ (**Figure 2a**). Caki 1 cells exhibited an increased sensitivity to $1,25(OH)_2D_3$-3-BE compared to A498 cells. In Caki 1 cells the growth inhibitory effect of $1,25(OH)_2D_3$-3-BE (10^{-6}M to 10^{-7}M) is more pronounced than in A498 cells. Additionally, equimolar doses of $1,25(OH)_2D_3$-3-BE and $1,25(OH)_2D_3$ demonstrated that $1,25(OH)_2D_3$-3-BE is more efficacious than $1,25(OH)_2D_3$ in eliciting inhibition of cell growth in Caki 1 cells (**Figure 2b**). These results suggest that irreversible linking of $1,25(OH)_2D_3$-3-BE into the hormone binding pocket of VDR in the target cells may be responsible for the enhanced antiproliferative activity of $1,25(OH)_2D_3$-3-BE versus $1,25(OH)_2D_3$ in kidney cancer cells.

Further support for the above hypothesis stems from our observation in an earlier study with prostate cancer cells. DU-145 prostate cancer cells are resistant to $1,25(OH)_2D_3$-mediated growth inhibition due to high levels of CYP enzymes that catabolize the hormone (26,27). However, treatment of DU-145 cells with $1,25(OH)_2D_3$ –3-BE resulted in strong growth inhibition suggesting unavailability of the compound inside the binding pocket to catabolizing enzymes (11).

$1,25(OH)_2D_3$-3-BE treatment induces apoptosis in A498 cells. The cellular mechanism(s) leading to growth inhibition by $1,25(OH)_2D_3$ are complex. In most cases $1,25(OH)_2D_3$ causes cells to arrest in the G_0/G_1 phase of the cell cycle (28). This effect is thought to be mediated by increased expression of the cyclin-dependent kinase (CDK) inhibitors p21 and p27, as well as other cell-cycle regulators (29). However, it has been reported that cellular growth inhibition mediated by $1,25(OH)_2D_3$ correlates with increased apoptosis in some studies. For example, it is reported that $1,25(OH)_2D_3$ induces apoptosis in LNCaP prostate cancer and MCF-7 breast cancer cells (30,31), but this result is not universal (32). Previously, we reported that $1,25(OH)_2D_3$ -3-BE induces apoptosis in PC-3 prostate cancer cells (11). Therefore, to determine whether $1,25(OH)_2D_3$ –3-BE inhibits A498 cell proliferation via apoptosis we measured apoptosis in A498 cells following $1,25(OH)_2D_3$ –3-BE treatment by FACS analysis.

In this assay the bottom left quadrant (LL) indicates the percent amount of live cells, the bottom right (LR) quadrant indicates the percent of early apoptotic cells, the upper right (UR) quadrant indicates the percent of late apoptotic cells, and the upper left (UL) quadrant indicates dead cells (**Figure 3**). It should be noted that the upper left quadrant is not considered as a

measure of apoptotic death as it indicates total cell death contributed by apoptosis, necrosis or any other means.

Results from this experiment show that 10^{-6}M of 1,25$(OH)_2D_3$ causes approximately 26.85% of the cells to undergo early apoptosis (LR), while an equimolar amount of 1,25$(OH)_2D_3$-3-BE causes 60.58% of the cells to undergo rapid apoptosis within 6 hours. These results are similar to our observation in PC-3 prostate cancer cells (11) and suggest 1,25$(OH)_2D_3$-3-BE is more efficacious in promoting apoptosis than 1,25$(OH)_2D_3$.

1,25$(OH)_2D_3$-3-BE inhibits Akt phophorylation in A498 cells. Akt (also known as protein kinase B, PKB) is a serine/threonine kinase which is activated by many signals in a phophatidylinositol-3'-kinase (PI3K)-dependent manner. (33,34). Akt is involved in a variety of normal and tumorigenic functions such as cell proliferation, growth and survival. As a result Akt has been an attractive target for cancer drug discovery. Akt activation is common in many malignant cells, while expression of PTEN (phosphatase and tensin homolog deleted on chromosome 10), a negative regulator of Akt signaling, is down-regulated. For example, Hara *et al.* screened forty five (45) tumor samples from renal cell carcinoma (RCC) patients and reported that phosphorylated Akt (p-Akt) expression increased significantly in comparison with that of the corresponding normal kidney tissue (35). PTEN expression inversely correlated with the p-Akt expression. These alterations were specific for clear cell type RCC, but not for papillary or chromophobe type RCC. In addition, these researchers observed that an Akt inhibitor induced apoptosis in KU19-20 and Caki-2 cells which have high Akt activity.

As described earlier, 1,25$(OH)_2D_3$-3-BE inhibits the growth of kidney cancer cells and induces apoptosis. To investigate the molecular mechanism of 1,25$(OH)_2D_3$-3-BE-induced apoptosis in A498 cells, we examined the activation status of Akt, the pro-survival kinase. Akt is activated by phosphorylation at Thr308 and Ser473 which is known to promote cell survival and proliferation. Therefore, we analyzed the activation status of Akt by immnunoblot analysis with an antibody specifically recognizing Ser473-phosphorylated Akt (pAkt) and an antibody recognizing total Akt (Akt) following overnight treatment of A498 cells with 1,25$(OH)_2D_3$ or 1,25$(OH)_2D_3$-3-BE. Results of this assay are shown in **Figure 4**. 1,25$(OH)_2D_3$-3-BE strongly reduced the level of pAkt. An equimolar concentration of 1,25$(OH)_2D_3$ also reduced Akt phosphorylation, but to a much lower degree than 1,25$(OH)_2D_3$-3-BE. In summary, results of this study demonstrate that apoptotic behavior of 1,25$(OH)_2D_3$-3-BE in kidney cancer cells may be mediated, at least partially, by signaling through the Akt pathway.

The cellular effects of 1,25$(OH)_2D_3$-3-BE in kidney cancer cells are VDR-dependent. 1,25$(OH)_2D_3$-3-BE is an alkylating agent and thus, in addition to alkylating VDR, it can potentially alkylate various cellular proteins non-specifically. The demonstration that cellular activities of 1,25$(OH)_2D_3$-3-BE are mediated by VDR is an essential facet of our studies to evaluate the mechanisms of the cellular properties of this compound. Several genes, including osteocalcin, osteopontin and 1,25-dihydroxyvitamin D_3-24-hydroxylase (CYP24) are known to be induced by 1,25$(OH)_2D_3$. In an earlier study we demonstrated that 1,25$(OH)_2D_3$-3-BE induces mRNA for osteocalcin and CYP24 in human keratinocytes (36). In the current study we evaluated whether 1,25$(OH)_2D_3$-3-BE is capable of modulating the expression of CYP24, similar to 1,25$(OH)_2D_3$, in A498 kidney cancer cells.

The results of this experiment, shown in **Figure 5**, demonstrate that treatment of A498 cells with 10^{-6}M to 10^{-9}M of 1,25$(OH)_2D_3$ strongly induces CYP24-message. In the case of 1,25$(OH)_2D_3$-3-BE, induction of CYP24 mRNA is observed at 10^{-6}M to 10^{-8}M, but there is almost no detectable induction of CYP24 mRNA at 10^{-9}M. This is in contrast with the equivalent dose of 1,25$(OH)_2D_3$. These results demonstrate a log scale difference in inducing the message for CYP24 between 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$-3-BE. It is noteworthy that we observed a similar difference between 25-OH-D_3-3-BE (the bromoacetate derivative of 25-OH-D_3) and 1,25$(OH)_2D_3$ in COS-7 cells transfected with a CYP24 reporter construct (12).

The CYP24 gene product catalyzes the introduction of a hydroxyl group at the 24-position in $1,25(OH)_2D_3$, followed by multiple oxidations of the side chain leading to calcitroic acid, the final catabolite that is excreted (37). Therefore, CYP24 is the initiator of the catabolic degradation of $1,25(OH)_2D_3$. We hypothesize that covalent attachment of $1,25(OH)_2D_3$-3-BE into the ligand binding pocket of VDR will decrease its catabolism. In essence it would require more $1,25(OH)_2D_3$-3-BE to induce the same level of CYP24 message as $1,25(OH)_2D_3$. Therefore, one log scale difference between concentrations of $1,25(OH)_2D_3$-3-BE and $1,25(OH)_2D_3$ to induce same level of CYP24-message is expected; and this is reflected in the results shown in **Figure 5**.

To further investigate the requirement for VDR in $1,25(OH)_2D_3$-3-BE action, we used the specific VDR antagonist, ZK159222 which has been shown to be effective in blocking VDR-mediated gene regulation (14). A498 cells were treated with either $1,25(OH)_2D_3$-3-BE or ZK 15922 alone or in combination, and the levels of CYP24 mRNA analyzed by RT-PCR. We observed a strong inhibition of CYP24 induction when ZK 159222 is co-administered with $1,25(OH)_2D_3$-3-BE in a 1:1 molar ratio demonstrating that $1,25(OH)_2D_3$-3-BE binds to and activates VDR. In an earlier study we demonstrated that ^{14}C-$1,25(OH)_2D_3$-3-BE specifically labeled VDR in extracts of ROS 17/2.8 bone cells and calf thymus nuclear cytosol, indicating that labeling by $1,25(OH)_2D_3$-3-BE is VDR-specific (38). Therefore, collectively these results strongly suggest that the cellular effects of $1,25(OH)_2D_3$-3-BE are related to specific affinity alkylation of VDR by $1,25(OH)_2D_3$-3-BE.

Protein and DNA alkylating compounds such as estramustine, lomustine, procarazine, busulfan, cyclophosphamide, chlorambucil, temozolomide, platinum coordination complexes etc. are important components in the standard cancer chemotherapeutic regimen. But the majority of these compounds are non target-specific and, produce significant side effects, particularly at pharmacological doses. On the other hand, affinity alkylating compounds such as $1,25(OH)_2D_3$–3-BE targets and covalently labels the hormone-binding site of VDR. Thus, this compound is expected to possess greater antiproliferative activity than $1,25(OH)_2D_3$ in cancer cells.

One concern regarding potential therapeutic use of $1,25(OH)_2D_3$-3-BE is its hydrolytic potential. Since it contains an ester bond, hydrolysis would produce $1,25(OH)_2D_3$ and bromoacetic acid. It could be argued that the growth-inhibitory property of $1,25(OH)_2D_3$–3-BE is due to $1,25(OH)_2D_3$ and/or bromoacetic acid, or a combination of the two. In an earlier study we demonstrated that antiproliferative property of $1,25(OH)_2D_3$-3-BE is due solely to its un-hydrolyzed and intact form, and equivalent amounts of $1,25(OH)_2D_3$ and bromoacetic acid have no effect on cellular growth (11). Therefore, we conclude that, even if $1,25(OH)_2D_3$-3-BE undergoes partial hydrolysis in a living system, the unhydrolyzed and intact form of $1,25(OH)_2D_3$-3-BE represents the biologically active form. It is also noteworthy that, while $1,25(OH)_2D_3$-3-BE produced strong antiproliferative and apoptotic effects, equivalent amounts of $1,25(OH)_2D_3$ failed to produce such effects. Therefore even if 'some' amount of $1,25(OH)_2D_3$ is produced by the hydrolysis of $1,25(OH)_2D_3$-3-BE, it should have minimal effect. Drugs containing hydrolysable bonds are fairly common. For example aspirin contains a hydrolysable ester bond, yet acetyl salicylate is the active principle of this drug, and not salicylic acid, its hydrolyzed form. In the case of $1,25(OH)_2D_3$-3-BE, both this molecule and its hydrolyzed product ($1,25(OH)_2D_3$) are biologically active, but $1,25(OH)_2D_3$-3-BE is a significantly stronger antiproliferative-apoptotic agent in rena carcinoma cells than $1,25(OH)_2D_3$, as delineated in this report.

During the past decade hundreds of vitamin D-analogs have been synthesized with the goal of obtaining a better antitumor/toxicity ratio and tumor-specific effects. Although a few of these analogs have successfully completed pre-clinical studies for several cancers, and at least one analog has shown strong effects in phase II trials for pancreatic and hepatocellular carcinomas (20, 21), the majority of these compounds have been proven to be of limited

therapeutic value due to toxicity. As a result new strategies for developing such analogs are required. As detailed in this communication we have developed 1,25$(OH)_2D_3$-3-BE, based on our knowledge about the interaction between 1,25$(OH)_2D_3$ and VDR-mediated cell-signaling processes. We have demonstrated that this compound possesses strong antiproliferative and apoptotic properties in kidney cancer cells. Therefore, 1,25$(OH)_2D_3$-3-BE has a strong translational potential as a therapeutic agent for kidney cancer.

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Figure Legends

Figure 1: Cartoon depicting the interaction of VDR with 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$-3-BE, and possible impact on such interaction on transcription and catabolism.

Figure 2: Cell-viability assay of A-498 (**Figure 2a**) and Caki-1 cells (**Figure 2b**). Briefly, A498 and Caki 1 cells were grown in manufacturer recommended media with FBS in 96-well plates (500-1000 cells/well) followed by treatment with various doses of 1,25$(OH)_2D_3$-3-BE or 1,25$(OH)_2D_3$ or ethanol (vehicle) (in media containing 5% FBS). The cells were dosed a total of three (3) times on alternate days. After seven (7) days, MTT solution (10 µl) was added to each well, and the plates were incubated at 37^0C for 3 hours followed by the addition of detergent solution (100 µl/well). The plates were incubated at 25^0C for 15 hr and then absorbance was read with a microplate reader. Absorbance from treated cells are plotted as percent of ethanol-control. There were eight replicates for each dose and control.

Figure 3: Annexin-PI-staining-FACS analysis of A-498 cells treated with 1,25$(OH)_2D_3$ or 1,25$(OH)_2D_3$ -3-BE. Briefly, A498 cells were grown to 60-70% confluence, and then were incubated (in media with 10% FBS) with 10^{-6}M of either 1,25$(OH)_2D_3$ or 1,25$(OH)_2D_3$-3-BE for 6 hours in a cell incubator. The control cells contained equivalent amount of ethanol as vehicle control. The cells were harvested, washed with PBS and then once with binding buffer. Cells were re-suspended in 100 ul of 1X binding buffer and then stained with Annexin-FITC and Propidium Iodide (PI) for 15 minutes in the dark, followed by dilution with 1X binding buffer. Fluorescence was measured in a FACS analyzer with FITC in the X-axis and PI in the Y-axis in log scale.

Figure 4: Effects of 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$-BE on phosphorylation of Akt in A498 cells. A498 cells were incubated with 5×10^{-7}M of 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$-BE or ethanol control for 24 hours and immunoblot analysis was used to asses the levels of phosphorylated Akt (p-Akt). The blot was stripped and re-probed for total Akt as a loading control. The results are representative of two independent experiments.

Figure 5: Comparison of dose dependent increase of Cyp 24 mRNA by 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$-BE in A498 cells. A498 cells were treated with ethanol control or the indicated doses of 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$-BE for 24 hours, total RNA prepared and Cyp 24 mRNA levels assessed by RT-PCR. Beta actin mRNA levels were determined for each sample as control.

Figure 6: Induction of Cyp 24 mRNA by $1,25(OH)_2D_3$-BE is blocked by a vitamin D receptor antagonist. A498 cells were treated with ethanol control, $1,25(OH)_2D_3$-BE (10^{-8}M) or a combination of $1,25(OH)_2D_3$-3-BE (10^{-8}M) and increasing amounts of ZK 159222 for 24 hours. (A) RT-PCR analysis was performed to assess Cyp 24 mRNA levels. Beta actin mRNA levels were determined for each sample as control. (B) Graphical representation of the band intensities in (A). Values represent the Cyp 24 band intensity normalized to the corresponding beta actin band intensity for each sample.

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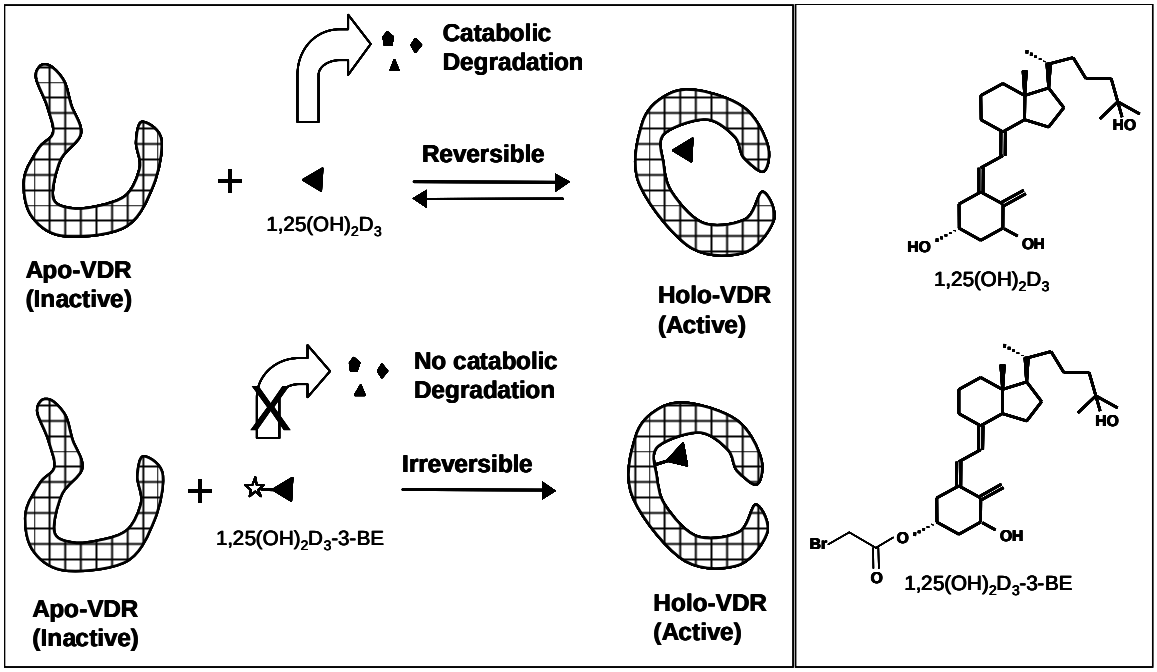
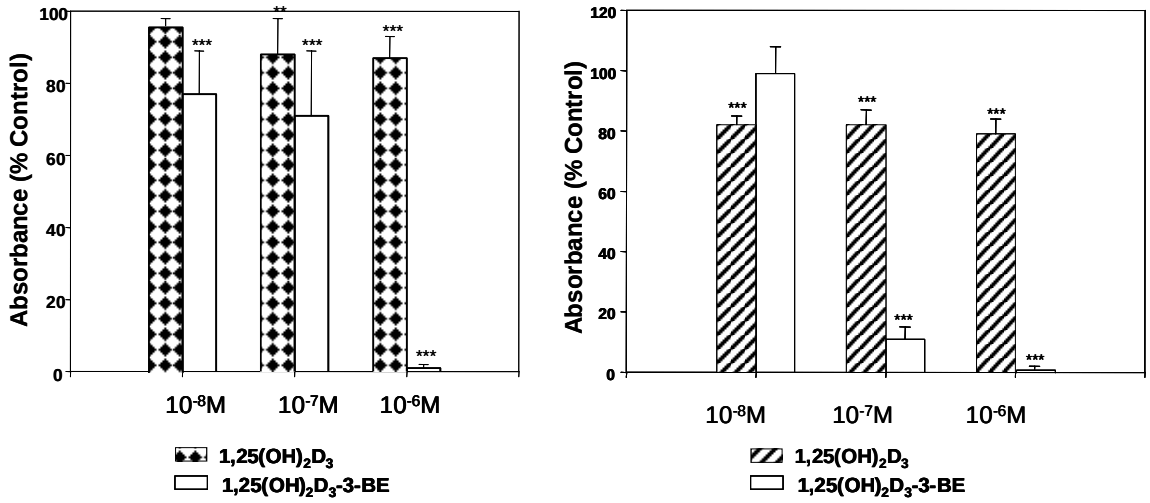


Figure 1

Figures 2a and 2b



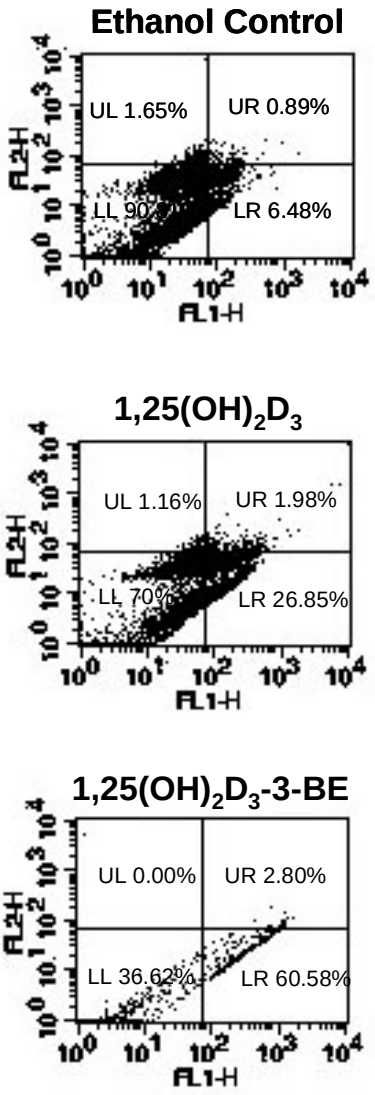


Figure 3

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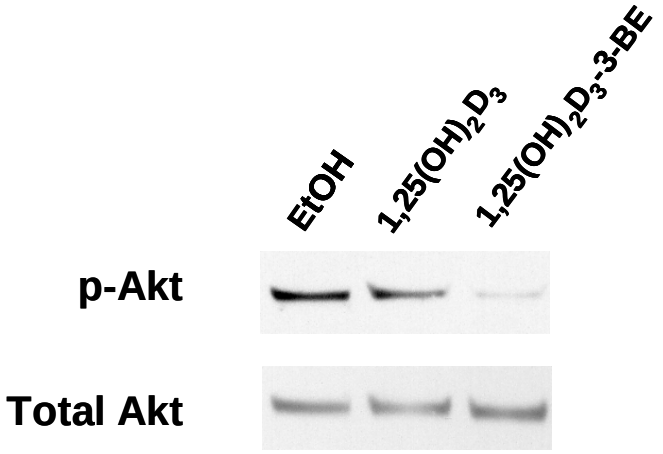

Figure 4

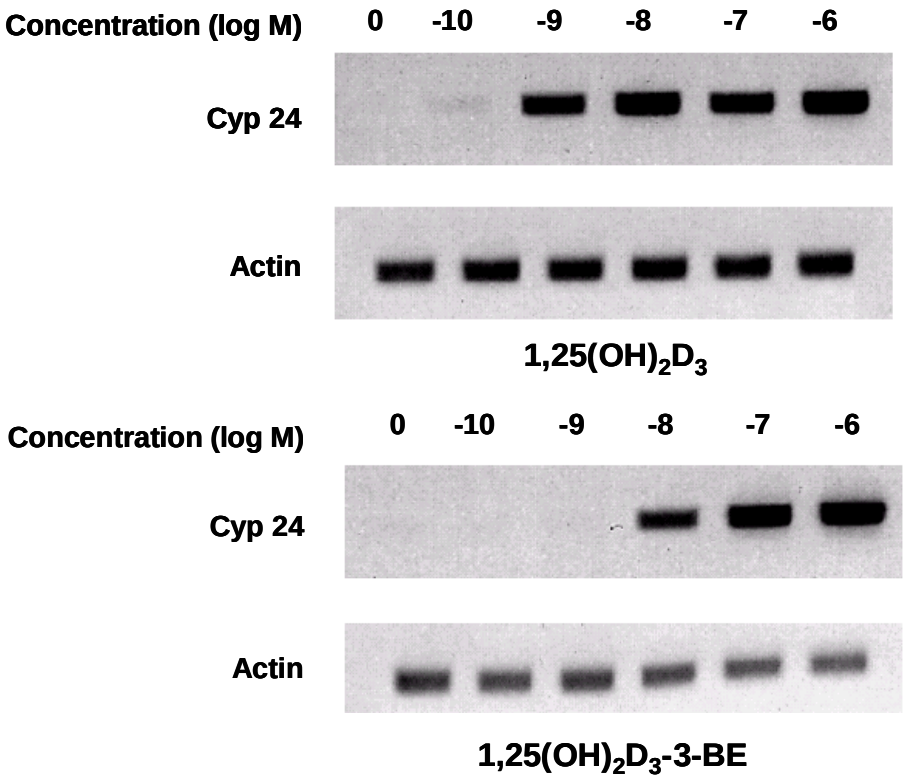

Figure 5

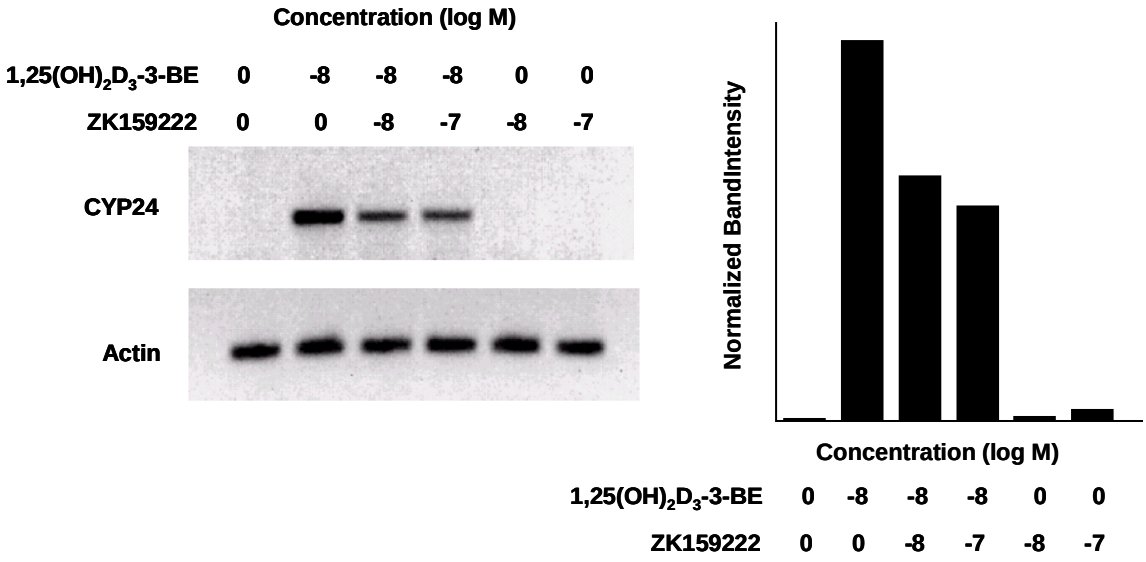

Figure 6